The assembly of lipid droplets and its relation to cellular insulin sensitivity

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Abstract

The assembly of lipid droplets is dependent on PtdIns(4,5)P_2 that activates PLD_1 (phospholipase D_1), which is important for the assembly process. ERK2 (extracellular-signal-regulated kinase 2) phosphorylates the motor protein dynein and sorts it to lipid droplets, allowing them to be transported on microtubules. Lipid droplets grow in size by fusion, which is dependent on dynein and the transfer on microtubules, and is catalysed by the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins SNAP-23 (23 kDa synaptosome-associated protein), syntaxin-5 and VAMP-4 (vesicle-associated protein 4). SNAP-23 is also involved in the insulin-dependent translocation of the glucose transporter GLUT4 to the plasma membrane. Fatty acids induce a missorting of SNAP-23, from the plasma membrane to the interior of the cell, resulting in cellular insulin resistance that can be overcome by increasing the levels of SNAP-23. The same missorting of SNAP-23 occurs *in vivo* in skeletal-muscle biopsies from patients with T2D (Type 2 diabetes). Moreover, there was a linear relation between the amount of SNAP-23 in the plasma membrane from human skeletal-muscles biopsies and the systemic insulin-sensitivity. Syntaxin-5 is low in T2D patients, which leads to a decrease in the insulin-dependent phosphorylation of Akt (also known as protein kinase B). Thus both SNAP-23 and syntaxin-5 are highly involved in the development of insulin resistance.

Introduction

Neutral lipids, such as triacylglycerols or cholesteryl esters, are stored in lipid droplets in the cytosol. This phenomenon is preserved throughout evolution and is present in most mammalian cells [1–4]. For many years, lipid droplets were considered to be only static fat depots. However, their role has recently been re-evaluated after observations that they have a complex surface [4], move in the microtubule network [5,6] and interact with other organelles such as mitochondria [7], peroxisomes [8] and the ER (endoplasmic reticulum) [9]. They are thus now regarded as highly dynamic organelles that play a role in several cellular processes.

Organization of the lipid droplet

Lipid droplets consist of a core of neutral lipids, surrounded by a monolayer of amphipathic lipids, such as phospholipids and unesterified cholesterol [2–4]. A number of proteins are also associated with this monolayer: the best described are the PAT-domain proteins, which include perilipin, ADFP (adipocyte differentiation-related protein), TIP47 (47 kDa tail-interacting protein), LSDP5 (lipid droplet storage protein 5) and S3-12. Perilipin is expressed only in adipose tissue and has a dual role: it protects the triacylglycerols from hydrolysis in its non-phosphorylated state and, in addition, promotes hydrolysis once phosphorylated (reviewed in [4]). ADFP has also been suggested to protect the turnover of triacylglycerols in lipid droplets: overexpression of ADFP in liver cells prevents fatty acids from entering into other metabolic pathways such as the formation of very low density lipoproteins ([10], reviewed in [3,4]).

Several other proteins have also been described in lipid droplets. They are involved in processes such as sorting/transport [e.g. Rab, ARF (ADP-ribosylation factor) and motor proteins] and turnover of lipids [e.g. ATGL (adipose triacylglycerol lipase) and its co-activator CGI-58, HSL (hormone-sensitive lipase), DGAT (diacylglycerol acyltransferase), acyl-CoA synthase and $cPLA_2$ (cytosolic phospholipase A_2)] (reviewed in [4]).

Assembly of lipid droplets (Figure 1)

Lipid droplets are formed from the microsomal membranes by a process that is dependent on triacylglycerol biosynthesis [11]. However, several other factors are also of importance for the assembly process, including PLD_1 (phospholipase D_1) [12,13] and PtdIns(4,5)P_2 (L. Li, B. Liu, L. Andersson, E. Lu and S.-O. Olofsson, unpublished work). PLD_1 catalyses the formation of phosphatidic acid, a lipid that is essential for lipid droplet assembly [12,13]. In addition, the absolute importance of PtdIns(4,5)P_2 for the assembly

Key words: fusion, insulin resistance, 23 kDa synaptosome-associated protein (SNAP-23), lipid droplet assembly, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (SNARE), syntaxin-5.

Abbreviations used: ADFP, adipocyte differentiation-related protein; ARF, ADP-ribosylation factor; $cPLA_2$, cytosolic phospholipase A_2; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; ERK2, extracellular-signal-regulated kinase 2; NSF, *N*-ethylmaleimide-sensitive factor; PLD_1, phospholipase D_1; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; α-SNAP, α-soluble NSF-attachment protein; SNAP-23, 23 kDa synaptosome-associated protein; T2D, Type 2 diabetes; VAMP-4, vesicle-associated protein 4.

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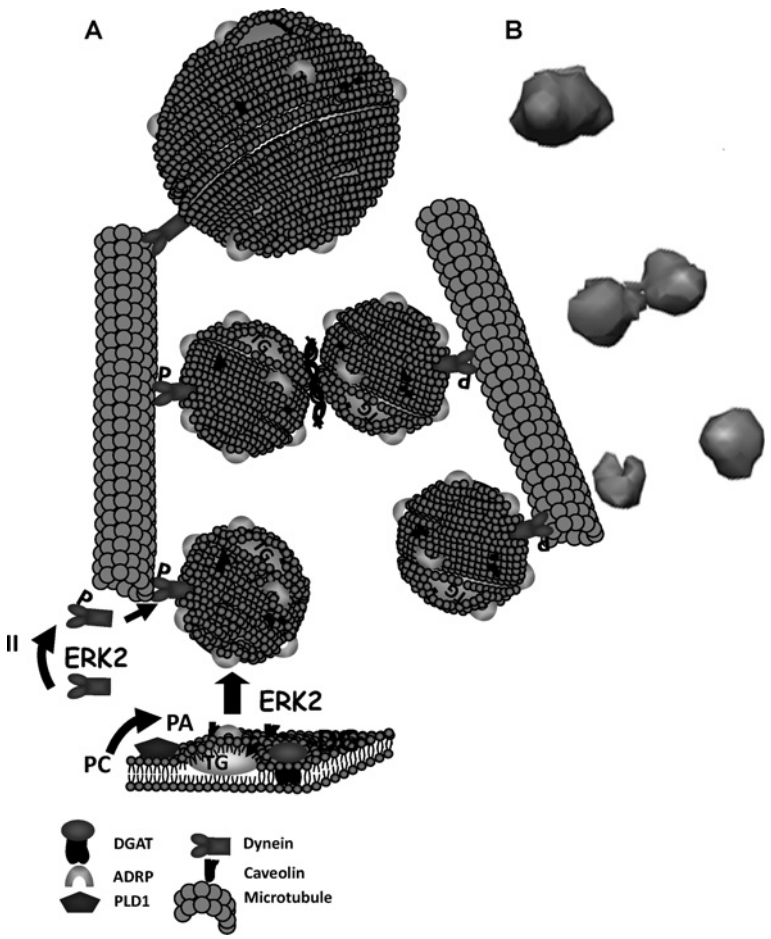
Figure 1 | **The assembly of lipid droplets**

The lipid droplets are formed from microsomal membranes. It is proposed that oiling out of triacylglycerols between the leaflets is essential in the assembly process. The assembly process is also dependent on PLD1 and ERK2. ERK2 phosphorylates dynein and sorts it to droplets. This allows for transfer on microtubules, which is essential for the fusion process that is involved in the increase in droplet size. The fusion process is catalysed by the SNARE proteins SNAP-23, syntaxin-5 and VAMP-4. (**A**) Schematic representation (the SNARE proteins are marked between fusing droplets). (**B**) Three-dimensional reconstruction of the assembly process between droplets from time lapse studies recorded by confocal microscopy.



of lipid droplets may, at least partly, be mediated through activation of PLD_1 because a PtdIns(4,5)P_2-binding site [a PH (pleckstrin homology) domain] in PLD_1 has been identified and PtdIns(4,5)P_2 is essential for PLD_1 activity [14,15]. Other PtdIns(4,5)P_2 targets that have been identified as important for the formation of lipid droplets include ARF1 (and proteins involved in the regulation of its activity) [16] and $cPLA_2$ [17].

We also showed that ERK2 (extracellular-signal-regulated kinase 2) is important for the formation of lipid droplets [13]. This enzyme phosphorylates the motor protein dynein and thereby sorts it to lipid droplets [13]. Our results clearly showed that dynein is essential for lipid droplet fusion (see below), but also indicated that dynein is involved in the initial formation of the droplet [13]. However, the details of this latter process have not been elucidated. One possibility is that dynein provides a 'pull force' that is necessary for the budding of droplets from the microsomal membrane.

The mechanism for the creation of cytosolic lipid droplets from the ER is not fully elucidated. One mechanism that was proposed several years ago (but without substantial experimental evidence) suggests that, upon formation, triacylglycerols are 'oiled out' between the leaflets of the microsomal membranes to form a lens that will become the core of the primordial lipid droplet. The rationale behind this model is that whereas the triacylglycerol precursors, diacylglycerols and acyl-CoA, are highly soluble in the cytosolic leaflet of the microsomal membrane, triacylglycerols have limited solubility in this leaflet, and are therefore forced into the hydrophobic part of the membrane (Figure 1) [3,11].

Growth of lipid droplets (Figure 1)

Newly synthesized lipid droplets are only 0.2 μm in diameter [11]. However, mature lipid droplets are much larger

(1–20 μm in diameter), indicating that they are able to grow after their formation. We demonstrated that they can grow by homotypic fusion, and that approx. 15 % of all droplets are engaged in this fusion process at a given time [6]. Although our results indicate that fusion between droplets is a quantitatively important process, we cannot rule out the importance of other mechanisms involved in lipid droplet growth. For example, it has been suggested that the surface of lipid droplets contain DGAT 2, which catalyses the conversion of diacylglycerols into triacylglycerols. Thus the droplets may acquire triacylglycerols by direct biosynthesis [18]. However, it could be argued that as DGAT 2 is known to span a bilayer twice [19], substantial adaptation of the enzyme would be required if it were to fit into the monolayer surface of the lipid droplet.

Fusion between lipid droplets is dependent on dynein, which promotes their transport on the microtubule network [6,13]. Recently, we also showed an involvement of the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins SNAP-23 (23 kDa synaptosome-associated protein), syntaxin-5 and VAMP-4 (vesicle-associated protein 4) [20] and proteins involved in their regulation, namely NSF (*N*-ethylmaleimide-sensitive factor) and α-SNAP (α-soluble NSF-attachment protein) [20].

The role of SNARE proteins and NSF and α-SNAP in fusion processes has primarily been investigated in the fusion process between transport vesicles and target membranes.

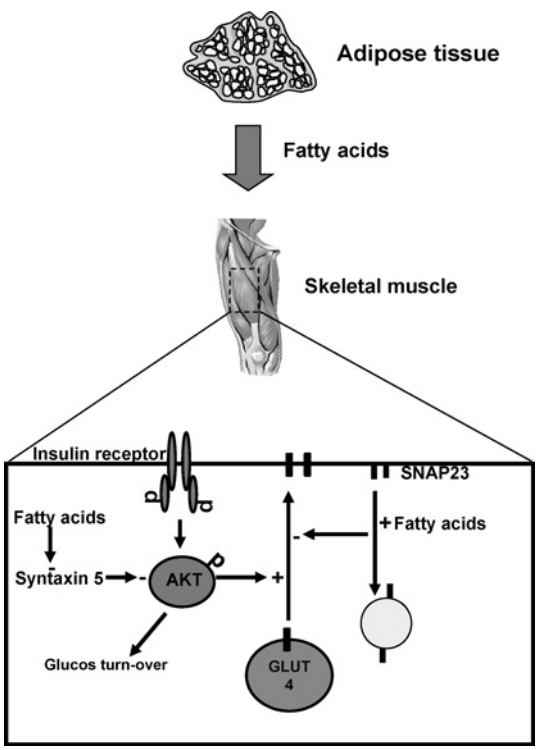
Central to the fusion process is the formation of a so-called four-helix bundle between α-helical domains (SNARE domains) present in the SNAREs. The formation of this four-helix bundle forces the membranes together, promoting their fusion. A detailed molecular model for this process has recently been proposed [21,22]. The stable four-helix bundle present after the completion of fusion is unwound by NSF (an ATPase) and its adaptor protein α-SNAP (for reviews, see [22–25]).

A transport vesicle is surrounded by a bilayer, whereas the lipid droplet surface is a monolayer; thus it is likely that there are differences between the fusion of vesicles and of lipid droplets. The stalk hypothesis has been proposed to describe the fusion process between bilayers [24]. We postulate that fusion between lipid droplets requires fewer steps and is complete at a stage equivalent to the creation of a 'fusion stalk', i.e. when the two outer monolayers of the bilayers have fused, and there is a continuum between the hydrophobic portions of the two membranes. For lipid droplets, this would correspond to a fusion of the monolayers surrounding the two droplets connecting the two hydrophobic cores [20].

It should be pointed out that the presence of SNARE proteins in lipid droplets and the described fusion process open up the possibility for interactions with other organelles. Thus a fusion between a lipid droplet and the outer monolayer of the bilayer around a peroxisome may theoretically result in a structure very similar to that suggested to be formed between these two organelles [8].

Figure 2 | Tentative roles for SNAP-23 and syntaxin-5 in the development of insulin-sensitivity

An increased amount of fatty acid, which reaches the skeletal muscle from, for example, the adipose tissue, results in a redistribution of SNAP-23 from the plasma membrane to the interior of the cell. This results in a decreased insulin-dependent translocation of GLUT4 to the plasma membrane, i.e. insulin resistance. Increased availability of fatty acids in the skeletal muscle decreases the expression of syntaxin-5, which leads to a decrease in insulin-dependent activation of Akt, i.e. to insulin resistance. Thus SNAP-23 and syntaxin-5 are targets by which different mechanism that affect the insulin-sensitivity in the cell can be reached.



Lipid droplets and insulin-sensitivity (Figure 2)

Insulin resistance is one of the most important metabolic diseases and a risk factor for the development of both cardiovascular diseases and T2D (Type 2 diabetes).

The glucose turnover in the skeletal muscles is of particular importance in the development of insulin resistance. The signalling of insulin, via its receptor, results both in an increased uptake and an increased utilization of glucose (glycolysis and oxidation as well as glycogen biosynthesis). The accumulation of triacylglycerols in skeletal muscles is highly related to the development of insulin resistance/T2D [26–29], but the mechanism is not yet clarified and the concept is complicated by the fact that highly insulin-sensitive athletes, such as marathon runners, have increased levels of triacylglycerols in their muscles [30]. This, and the fact that triacylglycerols stored in lipid droplets are very inert, argues against that the triacylglycerols as such are the reason for the

insulin resistance. Rather it has been proposed that metabolic products of triacylglycerols and released fatty acids are key factors in the development of the insulin resistance. Examples of such products are diacylglycerols, ceramides and partially oxidized fatty acids [31–34].

Of central importance for the insulin stimulation of the uptake of glucose is the translocation of the glucose transporter GLUT4 from intracellular storage to the plasma membrane. This process involves the fusion between GLUT4-specific transport vesicles and the plasma membrane. This fusion requires the SNARE proteins syntaxin-4, SNAP-23 and VAMP-2 (see for example [35]). Thus SNAP-23 is involved both in the fusion between lipid droplets and in the insulin-dependent translocation of the GLUT4 to the plasma membrane. We used the cardiomyocyte cell line HL-1 cells to investigate the process. Our results [20] demonstrated that fatty acids diverted SNAP-23 from the plasma membrane to the interior of the cell where it was found in lipid droplets. This resulted in an insulin resistance that could be overcome by increasing the amount of SNAP-23 in the cell. This opens up a new model by which triacylglycerols can influence the insulin-sensitivity, i.e. it is not the triacylglycerols themselves but proteins involved in their formation and processing that is of importance. We have recently confirmed the role of SNAP-23 in *in vivo* studies in which we compared skeletal-muscle biopsies from patients with T2D and controls (P. Boström, B. Falbe Vind, L. Andersson, L. Håverson, E. Larsson, J. Perman, J. Borén, H. Beck-Nielsen, K. Højlund and S.-O. Olofsson, unpublished work). We found that the amount of SNAP-23 in the plasma membrane of the skeletal-muscle biopsies was much lower in the patients with diabetes than in the controls. We also found a strong positive correlation between the amount of SNAP-23 in the plasma membrane and the systemic insulin-sensitivity measured by euglycaemic hyperinsulinaemic clamp. Thus the translocation of SNAP-23 between the plasma membrane and the interior of the skeletal muscle cell is an important mechanism for modifying, not only the local, but also the systemic insulin-sensitivity (P. Boström, B. Falbe Vind, L. Andersson, L. Håverson, E. Larsson, J. Perman, J. Borén, H. Beck-Nielsen, K. Højlund and S.-O. Olofsson, unpublished work).

Interestingly, the comparison of skeletal-muscle biopsies between patients with T2D and controls led us to identify syntaxin-5, which is also involved in the fusion between lipid droplets, as important for the development of insulin resistance (P. Boström, B. Falbe Vind, L. Andersson, L. Häverson, E. Larsson, J. Perman, J. Borén, H. Beck-Nielsen, K. Højlund and S.-O. Olofsson, unpublished work). Syntaxin-5 was low in the skeletal muscle from the patients with T2D and correlated with the insulin-dependent phosphorylation of Akt (also known as protein kinase B) as a measure of the insulin signal. Moreover, knockdown of syntaxin-5 in cultured human skeletal-muscle cells, as well as in a skeletal-muscle cell line, resulted in a decreased insulin-dependent phosphorylation of Akt. The reason seems to be that low levels of syntaxin-5 gave rise to increased levels of diacylglycerols in the cell. This in turn may be related to the observation that the promotion of formation [10] and fusion [36] of lipid droplets protects the hydrolysis of triacylglycerols and prevents degradation products from triacylglycerols entering into other metabolic pathways. Thus it is possible that low levels of syntaxin-5 have the opposite effect, allowing diacylglycerols to be formed from triacylglycerols and enter into other parts of the cell.

Thus two of the SNARE proteins involved in the formation of lipid droplets have central roles in the modulation of the insulin-sensitivity. Our results indicate that fatty acids have a central role in this process and we propose that SNAP-23 and syntaxin-5 are targets by which fatty acids from, for example, obese adipose tissue inhibit the insulin-dependent glucose turnover in the skeletal muscle, leading to systemic insulin resistance (Figure 2).

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